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Biochimica et Biophysica Acta 1455 (1999) 1–11

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Aberrations of ammonia metabolism in ornithine carbamoyltransferase-deficient spf-ash mice and their prevention by treatment with urea cycle intermediate amino acids and an ornithine aminotransferase inactivator

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Received 31 March 1999; received in revised form 8 June 1999; accepted 10 June 1999

Abstract

Sparse fur with abnormal skin and hair (spf-ash) mice are deficient in ornithine carbamoyltransferase (OCT) activity, but their OCT protein is kinetically normal. We administered ammonium chloride to spf-ash mice, in order to analyze ammonia metabolism and to find a rationale for the therapy of OCT deficiency. Ammonia concentration in the liver of spf-ash mice increased to a level much higher than in the control. Ammonium chloride injection caused an increase in ornithine (Orn) 5 min after injection and an increase in the sum of Orn, citrulline (Cit) and arginine (Arg) for at least 15 min in the liver of control mice, but no increase in Orn, Cit and Arg in the liver of spf-ash mice. Treatment of spf-ash mice with Arg 5–20 min prior to the injection of ammonium chloride kept the hepatic ammonia concentration at a level comparable to that without the load. A significant reciprocal relationship between ammonia and Orn concentrations in the liver of spf-ash mice 5 min after an ammonium chloride load with or without Arg strongly suggests that ammonia disposal is dependent on the supply of Orn. In spf-ash mice loaded with tryptone as a nitrogen source, Arg supplementation showed a dramatic decrease in urinary orotic acid excretion in a dose-dependent manner. Similar effects were observed with Cit and Orn at the same dose, and a long-lasting effect with an ornithine aminotransferase inactivator, 5-(fluoromethyl)ornithine, at a much lower dose. The rate of urea formation in liver perfused with ammonium chloride was lower in spf-ash mice than in controls, but with the addition of Orn to the medium it increased to a similar level in control and spf-ash mice. These results indicate that OCT is not saturated with Orn in vivo under physiological conditions and that the administration or enrichment of the urea cycle intermediate amino acids enhances the OCT reaction so that the ammonia metabolism of OCT-deficient spf-ash mice is at least partially normalized.

Keywords: 5-(Fluoromethyl)ornithine; Hyperammonemia; Ornithine aminotransferase; Ornithine carbamoyltransferase; Sparse fur with abnormal skin and hair mouse

Abbreviations: spf-ash, sparse fur with abnormal skin and hair; spf, sparse fur; OCT, ornithine carbamoyltransferase; OAT, ornithine aminotransferase; 5-FMOrn, 5-(fluoromethyl)ornithine

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0925-4439/99/$ – see front matter
PII: S0925-4439(99)00048-4

1. Introduction

Ornithine carbamoyltransferase (OCT) deficiency is the most frequent enzyme deficiency in the urea cycle [1]. The genetic defects and the enzyme abnormalities have been extensively studied [2,3]. Two kinds of OCT-deficient animal models, sparse fur (spf) and sparse fur with abnormal skin and hair (spf-ash) mice, have been analyzed [4–6] and used as model systems for gene therapy [6–9]. A mutation causing an amino acid substitution and kinetic abnormalities, such as a higher K_m value for ornithine (Orn) and a higher pH optimum, was found in spf mice [4,5]. The mutation in spf-ash mice causes splicing abnormalities and an amino acid substitution resulting in the decrease in enzyme protein (about 5% of control), which, however, has a normal K_m value for Orn [4,6]. Transgenic spf-ash mice carrying recombinant DNA composed of 1.3 kb of the promoter region of the rat OCT gene fused onto rat OCT cDNA [10] showed an OCT activity of about 10% and 30% of the control levels in the liver and small intestine, respectively. These mice appeared normal without having sparse fur [11]. They are, however, not completely normal, because they excrete a larger amount of orotic acid in the urine after nitrogen load and become labile after tick infection [12].

It is supposed that Orn is one of the most important regulatory factors for the operation of the urea cycle. Orn concentration in the liver changes in response to changes in urea synthesis [13–15]. Intraperitoneal injection of ammonium chloride to rats causes a transient increase in Orn concentration in the liver [14]. It is, however, not clear whether these changes in Orn concentration play a role in urea synthesis under physiological and pathological conditions. A number of papers have described that arginine (Arg) or Orn administration lowers blood ammonia and the mortality rate of animals intoxicated with ammonium salts or with liver damage [16–19]. Objections to these experiments are that the OCT step is not rate-limiting, because OCT activity is much higher than carbamoylphosphate synthetase I or argininosuccinate synthetase (ASS) activities.

An ornithine aminotransferase (OAT) inhibitor, 5-(fluoromethyl)ornithine (5-FMOrn), which causes the elevation of Orn concentration in the liver, plasma and brain [20] was reported to have a protective effect in animal models with hyperammonemia, but less in rats with portocaval shunts [21–23]. 5-FMOrn administered to OCT-deficient spf mice showed marked effects on hyperammonemia, orotic aciduria and abnormal behavior [24]. This is considered to be due to the supply of endogenous Orn to the abnormal OCT in spf mice which have a higher K_m value for Orn. In our experiments, we used spf-ash mice which have OCT with normal kinetic properties, and showed that even in the liver of spf-ash mice with only 5% of control OCT activity, supplementation of Orn has a marked therapeutic effect, indicating that OCT is not saturated in vivo with Orn.

A part of this study was presented at a meeting on nutritional and acid-base aspects of amino acid metabolism and published [25].

2. Materials and methods

2.1. Animals

Control male mice, OCT-deficient spf-ash hemizygous mice (X′/Y) and spf-ash mice with OCT transgene (transgenic spf-ash mice; X′/Y, +/−) from the C57BL strain [10,11] were used in the present study. In some experiments, we also used spf mice from the BALB/C strain. The mating programs were described by Shimada et al. [11]. Heterozygous female mice (X′/X) were mated with transgenic male mice (+/−), descendants of No. 94, described by Murakami et al. [10]. DNA diagnosis of spf-ash gene and OCT transgene was performed as described by Kobayashi et al. [26] using genomic DNA isolated by the method of Qi et al. [27].

2.2. Animal treatment

Mice were maintained on laboratory chow (24% protein) from Clea Japan and used for experiments at 60–70 days of age. After overnight starvation, they were subjected to intraperitoneal injections of 0.2 M ammonium chloride (2 mmol/kg body weight) between 10.00 and 11.00 in the experiments shown in Figs. 1–3, and additionally to 0.2 M neutralized Arg (4 mmol/kg) or 0.2 M sodium chloride (as control experiments) 5 min–2 h prior to ammonium chloride

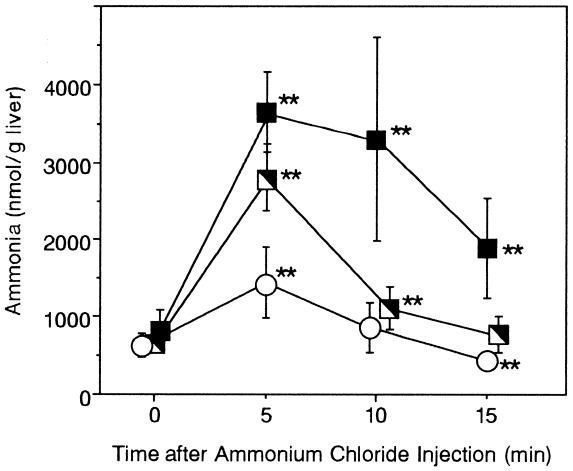

Fig. 1. Changes in ammonia concentration in the liver of control (○), spf-ash (■) and transgenic spf-ash (◥◣) mice after ammonium chloride injection. Mice (60–70 days old) were starved overnight. Ammonium chloride solution (0.2 M; 2 mmol/kg body weight) was intraperitoneally injected to control, spf-ash and transgenic spf-ash mice at time 0. At the times indicated, liver was taken out and freeze-clamped as quickly as possible. Values are presented as means ± S.D. Numbers of animals are 17, 9, 12, and 7 for 0, 5, 10, and 15 min of control mice; 23, 7, 10, and 6 for spf-ash mice; 6, 11, 12, and 5 for transgenic spf-ash mice. Statistically significant differences from the 0 min value of each group are $*P < 0.05$ and $**P < 0.01$.

injection in the experiments shown in Fig. 4. Five, 10 or 15 min after ammonium chloride injection, the mice were killed by cervical dislocation. For amino acid and ammonia analysis, the liver was taken out and freeze-clamped as quickly as possible in a solid CO_2/acetone mixture. For the experiments shown in Figs. 6 and 7, 1 g of tryptone (Difco Laboratories, Detroit, MI, USA) was dissolved in 3 ml of water, or 0.05, 0.1, 0.2 or 0.4 M neutralized Arg, 0.2 M neutralized Orn or 0.2 M citrulline (Cit). Amounts of tryptone and amino acids supplemented were calculated from the volume change by the addition of tryptone powder. The tryptone solution (20 ml/kg body weight), 5.6 g/kg or 46 mmol equivalent of amino acids/kg, was administered to spf-ash mice through a gastric tube between 9.00 and 10.00. Then, the mice were housed individually in metabolic cages for 2 h, to collect urine. Blood was taken by heart puncture under ethylether anesthesia at the times indicated in the figure. In the experiments shown in Figs. 6 and 7, 5-FMOrn was administered intraperitoneally at a dose of 20 mg/kg (0.1 mmol/kg) twice at 9.00 and 18.00 1 day before the tryptone load. This study was carried out according to the Guide for Animal Experimentation, Faculty of Medicine, Kagoshima University.

2.3. Liver perfusion

Liver perfusion was performed essentially as described by Nakajima et al. [28]. After anesthesia with an intraperitoneal injection of sodium pentobarbital (0.05 mg/g), liver was perfused in situ with Krebs-Henseleit bicarbonate buffer (pH 7.4, 36°C, saturated with 95% oxygen and 5% carbon dioxide) in a noncirculating system [29]. A Clark-type oxygen

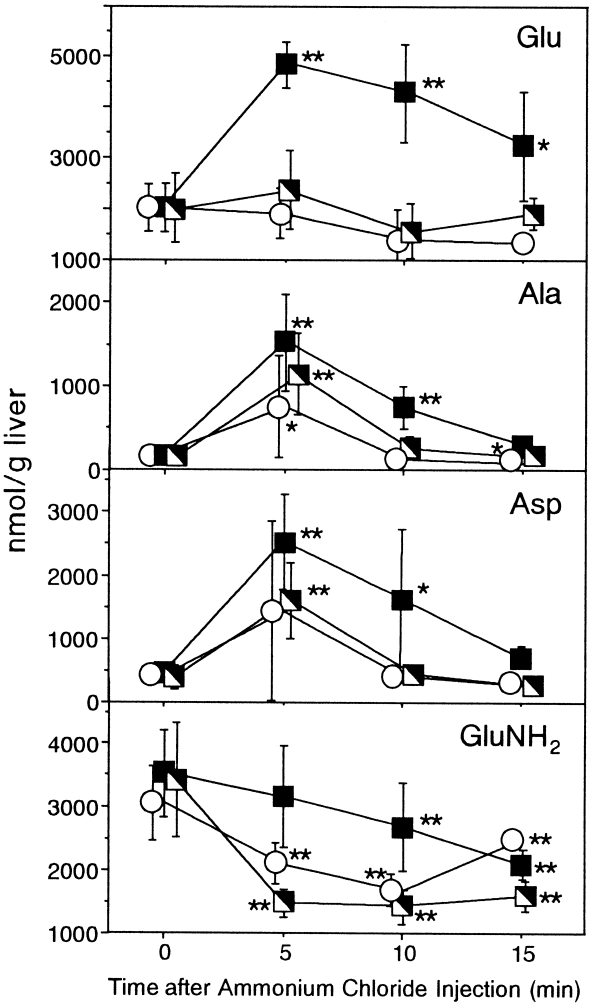

Fig. 2. Changes in Glu, Ala, Asp and $GluNH_2$ concentrations in the liver of control (○), spf-ash (■) and transgenic spf-ash (◥◣) mice after ammonium chloride injection. Experimental details are as described in the legend to Fig. 1.

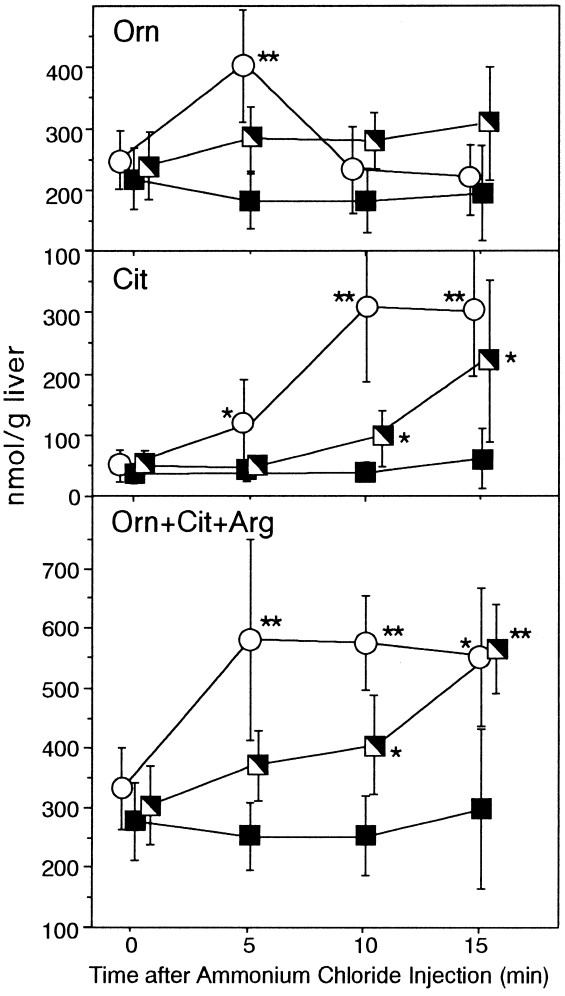

Fig. 3. Changes in concentrations of Orn, Cit and the sum of Orn, Cit and Arg in the liver of control (○), spf-ash (■) and transgenic spf-ash (◥) mice after ammonium chloride injection. Experimental details are as described in the legend to Fig. 1.

electrode (Instech, model 125/05) was used to measure oxygen concentration of the effluent perfusate. Perfusate and substrate were pumped into the liver through a cannula inserted in the portal vein, and the effluent perfusate was collected via a cannula placed in the inferior vena cava. The liver was perfused at a constant flow rate of 6–8 ml/min. Perfusion was considered successful if the liver did not swell or leak perfusate, and if oxygen consumption was stable. Ammonium chloride and L-ornithine-monohydrochloride (Orn) were dissolved in Krebs-Henseleit bicarbonate buffer to a final concentration of 1 mM.

2.4. Assay methods

Freeze-clamped livers were pulverized in liquid nitrogen, and the resultant powder was homogenized with 4 vols. of 3% sulfosalicylic acid. After neutralization with 1 M sodium bicarbonate, ammonia concentration in the sulfosalicylic acid extract was assayed by flow injection analysis as described by Svensson and Anfält [30]. Ammonia was assayed by flow injection analysis with plasma separated from heparinized blood. Amino acid concentrations in the salicylic acid extract were determined with a Hitachi 835 Amino Acid Analyzer (Hitachi, Japan). Urinary orotic acid and creatinine were measured by the methods of Kesner et al. [31] and Bonsnes and Taussky [32], respectively. Urea was assayed with an enzymatic method [33].

2.5. Materials

5-FMOrn was synthesized as described by Daune et al. [20]. Other chemicals were of analytical grade from Nacalai tesque (Kyoto).

2.6. Statistical methods

All data were analyzed using Student's *t*-test for unpaired samples. All values are expressed as means ± S.D.

3. Results and discussion

3.1. Changes in ammonia and amino acid concentrations in the liver of control, spf-ash and transgenic spf-ash mice after intraperitoneal injection of ammonium chloride

To test the ammonia metabolism of OCT-deficient mice and the effect of the OCT transgene, we injected an ammonium chloride solution (2 mmol/kg body weight) intraperitoneally to the overnight-fasted mice, and measured the ammonia concentration in the liver 5, 10 and 15 min after the injection. The 0 min ammonia level (without injection) of spf-ash mice was significantly higher than the values of control and transgenic spf-ash mice (816 ± 268 vs. 638 ± 150 and 651 ± 103 nmol/g liver; $P < 0.05$). As

shown in Fig. 1, ammonium chloride injection to control mice caused a significant increase in liver ammonia 5 min after injection, which then decreased to a level lower than the 0 min value. In the case of OCT-deficient spf-ash mice, the hepatic ammonia level increased at 5 min to a level about 5 times the 0 min value. Ammonia remained high until 10 min, then decreased to reach at 15 min a level still 2 times higher than the 0 min value. In the case of the transgenic spf-ash mice carrying rat OCT transgene, the hepatic ammonia increased at 5 min to a level much higher than the control value, but lower than the level of spf-ash mice. It decreased to the 0 min level at 15 min. These results indicate that the ammonia metabolism of OCT-deficient spf-ash mice is defective and that the OCT transgene is not sufficient to completely normalize the disturbed ammonia metabolism of spf-ash mice.

Next we measured the amino acid concentrations in the liver of the mice (Figs. 2 and 3). No significant differences in hepatic amino acid concentrations were found between the groups at 0 min, except that the glutamine ($GluNH_2$) concentration was higher in spf-ash mice than in control mice (3540 ± 692 vs. 3070 ± 586 nmol/g liver; $P < 0.05$). Orn and Cit concentrations were lower in spf-ash mice than in control mice at 0 min (Orn, 218 ± 49 vs. 249 ± 46 nmol/g, $P < 0.05$; Cit, 36 ± 17 vs. 54 ± 23 nmol/g, $P < 0.01$).

After the ammonium chloride injection, no significant changes in glutamate (Glu) were observed in the liver of control and transgenic spf-ash mice, while a marked increase in Glu was found in spf-ash mice. The levels were more than 2 times of the 0 min value at 5 and 10 min, and slightly decreased at 15 min, but were still much higher than at 0 min. The hepatic alanine (Ala) and aspartate (Asp) levels changed similarly after the ammonia administration; about 5-fold increases were seen in spf-ash and the transgenic spf-ash mice at 5 min, which decreased to the 0 min level of control and transgenic spf-ash mice at 10 min. The levels in spf-ash mice at 10 min were significantly higher than the 0 min value. The Ala level at 15 min of control mice was significantly lower than the 0 min value. It was unique that the $GluNH_2$ concentrations decreased after ammonium chloride injection. The decreases were more marked in control and transgenic spf-ash mice than in spf-ash mice; the levels were significantly lower at 5 min than the 0 min value and stayed low thereafter. In the case of spf-ash mice, $GluNH_2$ decreased gradually and was significantly lower at 10 and 15 min than at 0 min. These results suggest that Ala and Asp are transient ammonia acceptors under the conditions of ammonium salt load, not only in OCT-deficient mice, but also in control mice, while Glu is a reservoir for an excess of ammonia in the OCT-deficient mice. $GluNH_2$ is known to be degraded in the periportal region of the hepatic lobulus and synthesized in the pericentral region [34], and its degradation is accelerated in the presence of ammonia [35]. The degradation of $GluNH_2$ may be active both in control and spf-ash mice, although the simultaneous synthesis is probably more active in OCT-deficient spf-ash mice because the sustained high concentration of ammonia in spf-ash mice accelerates $GluNH_2$ synthesis.

We measured the concentrations of the urea cycle intermediate amino acids (Fig. 3). As stated above, the Orn and Cit concentrations at 0 min were significantly lower in spf-ash mice than in controls. The ammonium chloride injection to control mice caused an increase in Orn at 5 min, which then decreased to the 0 min level at 10 min. The Cit concentration markedly increased after the Orn concentration had decreased, and remained high until 15 min. The Arg level was increased 2-fold from 28 ± 10 to 58 ± 21 nmol/g ($P < 0.01$) at 5 min, and then decreased to the basal level at 10 min (data not shown). As a result, the sum of Orn+Cit+Arg concentrations increased about 1.7-fold at 5 min and remained almost constant till 15 min. In the case of OCT-deficient spf-ash mice, none of the three urea cycle intermediate amino acids was increased. Not only spf-ash mice, but also spf mice which are also OCT-deficient, having a kinetically mutant OCT, showed similarly no increase in the urea cycle intermediate amino acids after the intraperitoneal injection of ammonium chloride (data not shown). In contrast, the transgenic spf-ash mice showed no significant transient increases in Orn and Arg but a gradual increase in Cit. As a result, the sum of Orn+Cit+Arg was increased to the level of the control mice at 15 min.

In 1978, Saheki et al. [14] described that the intraperitoneal injection of ammonium chloride in rats caused a transient increase in hepatic Orn. Similar

phenomena were observed in the control mice in the present study. One difference between rats and mice was that the increase in Cit was prominent in mice, but very weak in rats. It suggests that the ASS step is more rate-limiting in mice than in rats. One of the most important results shown in the present study is that no increase in Orn or Orn+Cit+Arg was observed in OCT-deficient mice including spf and spf-ash. This suggests that OCT is involved in the increase in the hepatic Orn after ammonium salt load. One possibility is that the conversion of Orn to Cit causes the increase in urea cycle intermediate amino acids, because OAT is the major degradation enzyme of the urea cycle intermediate amino acids, and therefore, the conversion of Orn to Cit prevents Orn from being degraded. However, this cannot be the case, because after the ammonia salt load Orn concentrations increased before the increase in Cit. Another possibility is that an increase in Arg may be the first event, although Arg is immediately converted to Orn and therefore it is difficult to observe a significant increase in Arg. Arg is synthesized from Cit, which is synthesized in the small intestine from Glu via glutamic semialdehyde and Orn. OCT is also involved in this pathway. The OCT activity in the small intestine of spf-ash mice is 5% of the control, while it is 30% in the small intestine of the transgenic spf-ash mice. As Saheki et al. reported previously [14], Orn is very low and not increased in the perfused liver, supplemented with ammonium salt, suggesting that other organs may be involved in the increase in the urea cycle intermediate amino acids after ammonia salt load. This suggests the importance of OCT in the small intestine. The origin of the increased hepatic Orn and the way to increase the Orn concentration remain to be elucidated. Other questions are whether the increase in the urea cycle intermediate amino acids plays a role in the ammonia metabolism in the liver, and if it does, whether the supplementation of the urea cycle intermediate amino acids helps to dispose of ammonia in the liver of spf-ash mice. To test this, we supplied Arg intraperitoneally to spf-ash mice before the ammonium chloride load.

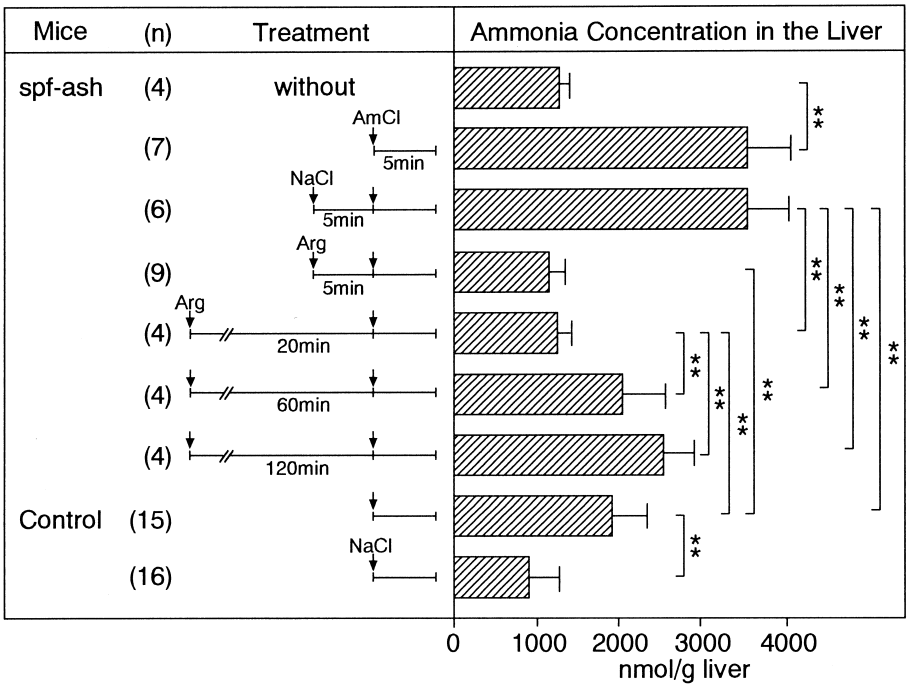

Fig. 4. Effect of pretreatment with Arg on ammonia concentration in the liver of spf-ash mice injected with ammonium chloride (AmCl). Spf-ash mice were treated with or without intraperitoneal injection of 0.2 M Arg (4 mmol/kg) or with 0.16 M sodium chloride (NaCl; 3.2 mmol/kg) 5 min–2 h prior to AmCl injection. Five minutes after AmCl injection, liver was taken out to measure ammonia concentration. Values are presented as mean ± S.D. Numbers of animals (n) are shown in parentheses. Statistically significant differences between the groups are $^{**}P < 0.01$.

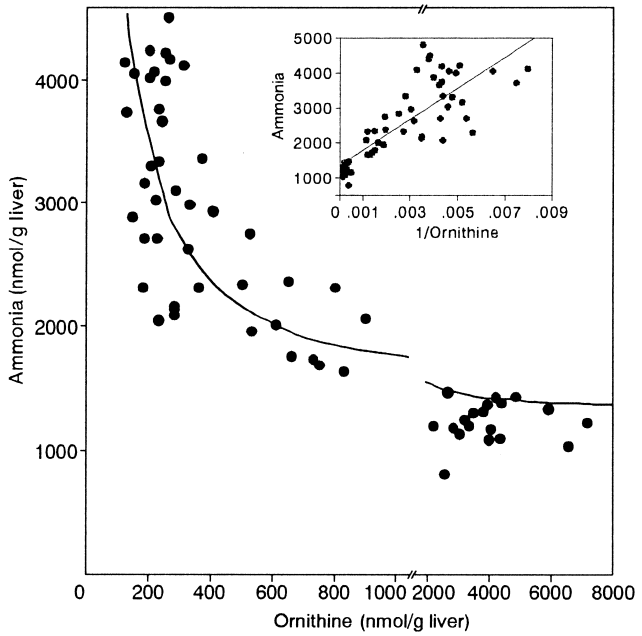

Fig. 5. Relationship between ammonia and ornithine concentrations in the liver of spf-ash mice 5 min after ammonium chloride load; effect of pretreatment with arginine. Data on ammonia and ornithine concentrations obtained in the experiments shown in Fig. 4 were plotted in this figure. The insert shows the relation between ammonia concentration (nmol/g liver; Y) and reciprocal number of ornithine concentration (g liver/nmol; X). The regression equation was as follows: $Y = 1320 + 444\,000X$. The correlation coefficient was $r^2 = 0.68$ ($P < 0.05$). The curve in the main figure was drawn according to the equation.

3.2. *Effect of arginine supplementation prior to ammonium chloride injection on ammonia concentration in the liver*

As shown in Fig. 4, pretreatment with Arg (4 mmol/kg body weight) 5–20 min prior to the ammonium chloride injection prevented the increase in ammonia in the liver of spf-ash mice at 5 min after ammonium chloride load. The Arg effect decreased if the time elapsing between Arg administration and ammonium chloride load increased, suggesting a rapid turnover of Arg and Orn. Under these conditions, the Orn concentration in the liver decreased with time. Fig. 5 represents relations between ammonia and Orn concentrations in the liver of spf-ash mice 5 min after ammonium chloride load with and without Arg pretreatment. When the former values were plotted versus the reciprocals of the latter values, we obtained a straight line, as shown in the insert of Fig. 5, indicating a significant linear relationship ($r^2 = 0.68$ and $P < 0.05$) between ammonia and the reciprocal of ornithine levels. A significant ammonia-decreasing effect was observed with ornithine concentrations at 400–600 nmol/g liver, which is about the same level of control mice after ammonium chloride load (Fig. 3). It is also noteworthy that the K_m value of OCT for ornithine is about 0.4 mM [4,36]. These results strongly suggest that the ability to dispose of ammonia is dependent on the supply of Orn, and that the increase in Orn after the ammonium chloride challenge has a physiological significance.

3.3. *Effect of arginine, ornithine, citrulline and 5-(fluoromethyl)ornithine on orotic acid excretion and plasma ammonia following oral administration of tryptone to spf-ash mice*

To further confirm the effect of the urea cycle intermediate amino acids on the ammonia metabolism of OCT-deficient spf-ash mice, we administered tryptone (5.6 g/kg body weight) with or without Arg via a gastric tube, and measured orotic acid excretion in the urine collected for 2 h. The orotic acid excretion in OCT-deficient mice was markedly decreased by the introduction of OCT transgene [11,12,37,38], or by viral vector-mediated gene therapy [7,9]. The orotic acid excretion decreased dose-dependently by the Arg supplementation at doses between 0.8 and 6.4 mmol/kg body weight (data not shown). The Arg at a dose of 0.8 mmol/kg caused a significant decrease in urinary orotic acid excretion ($P < 0.01$). We achieved the lowest orotic acid excretion with Arg at 6.4 mmol/kg. The lowest value was almost similar to that of the transgenic spf-ash mice with tryptone load without Arg, and was still higher than control values [12]. Almost the same effects were obtained by the supplementation with the same amount of Cit and Orn, as shown in Fig. 6.

We tested 5-FMOrn, a selective inactivator of OAT introduced by Daune et al. [20], on orotic acid excretion. 5-FMOrn at a dose of 20 mg/kg (0.1 mmol/kg) was administered intraperitoneally twice at 9.00 and 18.00 h 1 day before the tryptone load. A dose of 5-FMOrn lower by a factor of 1/30 of the most effective dose of Arg caused almost the same effect as Arg on orotic acid excretion. Its long-

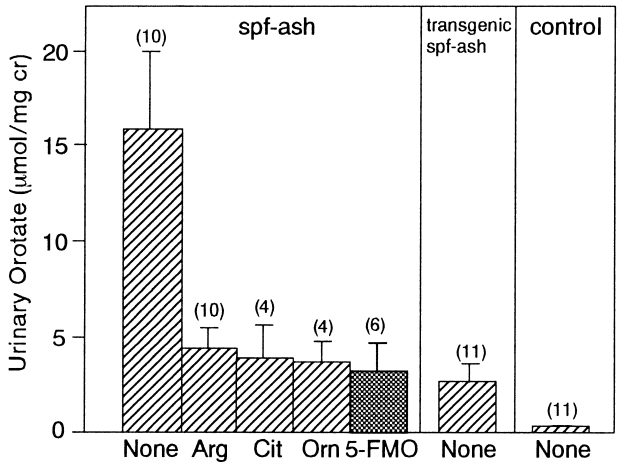

Fig. 6. Effect of Arg, Cit and Orn supplementation and pretreatment with 5-FMOrn on urinary orotic acid excretion in spf-ash mice following oral administration of tryptone. Tryptone was dissolved in water (none), or in 0.2 M neutralized Arg, Cit and Orn solution, and administered per os. Amount of amino acid supplemented was 3.2 mmol/kg. 5-FMOrn was injected intraperitoneally twice 1 day before the experimental day, and tryptone solution in water was administered, as described in Section 2. The mice were housed individually in a metabolic cages for 2 h, to collect urine. Data were expressed as μmol orotic acid/mg creatinine (cr). Data on transgenic spf-ash and control mice [12] are also presented.

lasting effect was remarkable. Because 5-FMOrn inactivates OAT, it causes a dramatic increase in Orn in the liver and other organs [20]. In the present study, Orn, Cit and Arg concentrations in the liver of spf-ash mice with 5-FMOrn treatment at 10.00 h on the day of the experiments were 4000 ± 550, 149 ± 44 and 53 ± 15 nmol/g liver, respectively (without ammonium chloride load, $n = 5$). Those in the plasma without and with 5-FMOrn were 16 ± 4 and 835 ± 396 nmol/ml for Orn, 10 ± 3 and 29 ± 14 nmol/ml for Cit, and 34 ± 7 and 46 ± 13 nmol/ml for Arg ($n = 5$). It is also noteworthy that not only Orn, but also Cit and Arg were elevated in the liver and plasma due to 5-FMOrn, although the difference was not significant for plasma Arg. In the case of the transgenic spf-ash mice, administration of 5-FMOrn decreased the orotic acid excretion after tryptone load from 2950 ± 1420 to 470 ± 150 nmol/mg creatinine, which is within the normal range [12].

Next we measured plasma ammonia levels of spf-ash mice following oral tryptone administration and tested the effect of 5-FMOrn. As shown in Fig. 7, the plasma ammonia level was elevated following the nitrogen load; the peak (781 ± 96 nmol/ml) was at 1 h after the administration, and the level was not normalized at 2 h. When the mice were pretreated with 5-FMOrn as described above, the plasma ammonia (106 ± 22 nmol/ml) was not significantly different from the value before the nitrogen load without 5-FMOrn (97 ± 69 nmol/ml). It was, however, still higher than the value of control mice with the tryptone load (55 ± 12 nmol/ml).

All these results suggest that the defective OCT activity of spf-ash mice is not the only rate-limiting parameter. The amount of Orn, or the urea cycle intermediate amino acids, limits the ability of the liver to detoxify ammonia. These considerations are important for the treatment of OCT deficiency.

3.4. *Urea synthesis from ammonium chloride as a substrate in the perfused liver of control and spf-ash mice*

To further test the rate-limiting factors of urea synthesis in the liver of OCT-deficient spf-ash mice, we performed liver perfusion experiments and measured the rate of urea synthesis with ammonium chloride as a substrate for the urea cycle in the ab-

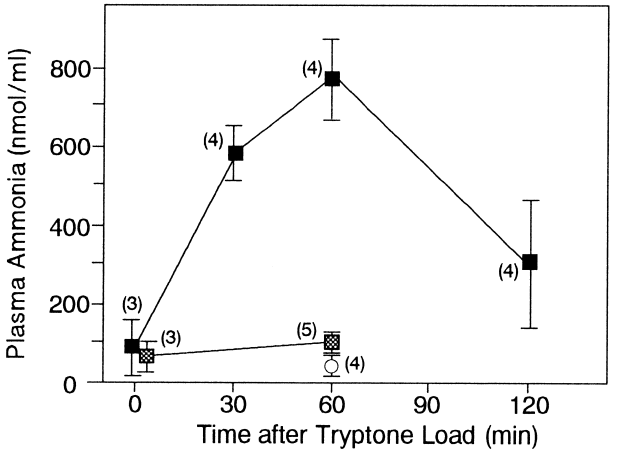

Fig. 7. Plasma ammonia concentration of spf-ash mice intragastrically loaded with tryptone and effect of 5-FMOrn) pretreatment. Experimental procedures are described in Section 2. Tryptone was administered at time 0. At the times indicated, blood was taken by heart puncture under ethylether anesthesia. Results of control mice receiving 0.2 M NaCl (4 mmol/kg) are shown with an open circle, and spf-ash mice administered 0.16 M NaCl (1.6 mmol/kg) and 5-FMOrn (0.1 mmol/kg) with closed and shaded squares, respectively. Values are presented as means ± S.D. Numbers of animals are shown in parentheses.

sence and presence of external Orn. The rate of urea synthesis in perfused liver was determined by measuring urea in the perfusate under flow-through conditions. As shown in Fig. 8A, addition of 1 mM ammonium chloride following 10 min preperfusion without external substrates caused an increase in urea synthesis in the livers from control, spf-ash and transgenic spf-ash mice. After the initial increase in the rate of urea formation for about 4 min, it further gradually increased with time in control mice and, to a lesser degree, in transgenic spf-ash mice, but it did not increase in the liver of spf-ash mice. Ten min after addition of ammonium chloride to the perfusion medium, there were significant differences in the rate of urea synthesis between the groups. The addition of 1 mM Orn increased the rate of urea formation, which was not different between the groups (Fig. 8A). Withdrawal of Orn from the perfusion medium caused again a significant difference in the rate between the groups. Withdrawal of ammonium chloride caused first a rapid and then a slow decrease in the rate in each group, and there was no difference in the rate between the groups.

When ammonium chloride and Orn were added to the medium from the beginning, as shown in Fig. 8B, a gradual increase in the rates of urea formation with time was observed not only in control but also in spf-ash mice, and no difference in the rates between the groups was found. The gradual increases in the rates of urea formation may result from changes in *N*-acetylglutamate or Orn which are well-known stimulators of urea formation in perfused liver and in liver slices [39–41]. Orn concentration in the perfused liver in the presence of ammonium chloride was at a similar level in control and spf-ash mice, at 42 ± 22 and 36 ± 11 nmol/g liver, respectively. A change in *N*-acetylglutamate concentration is considered to be the cause of the gradual increase in the rate of urea formation in the experiments shown in Fig. 8B, because 1 mM Orn was added to the perfusion medium. Saheki et al. [14] showed that the concentration of *N*-acetylglutamate of perfused rat liver increased during perfusion with ammonium chloride, although Orn did not. In the experiments shown in Fig. 8A, the rate of urea formation in spf-ash mice did not show a gradual increase with time. It is because the defective OCT step with low Orn would be rate-limiting even though *N*-acetylglutamate concentration may have

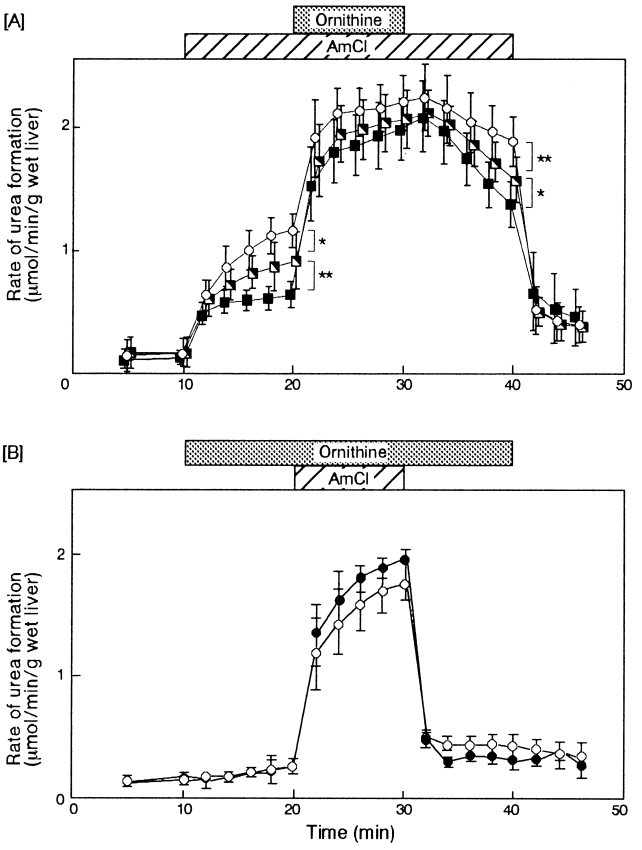

Fig. 8. Urea formation from ammonium chloride with or without ornithine in the perfused liver of control (○), transgenic spf-ash (◥) and spf-ash (■) mice. The liver perfusion system and the procedures are described in Section 2. The abscissa shows the perfusion time. The ordinate shows the rate of urea formation. 1 mM ammonium chloride (AmCl) and 1 mM ornithine were present in the medium during the period indicated. Numbers of animals were 8, 4 and 7 for control, transgenic spf-ash and spf-ash mice in A and 5 and 4 for control and spf-ash mice in B, respectively. Statistically significant differences between the groups at the times indicated (20 and 40 min) are $^{*}P < 0.05$ and $^{**}P < 0.01$.

increased. Only after the addition of Orn does any step other than the OCT reaction become rate-limiting.

The results show that addition of 1 mM Orn caused no difference in the rate of urea formation between control and spf-ash mice. This indicates that under the conditions of Orn supplementation, the OCT step is not rate-limiting for urea formation, even though spf-ash mice have only 5% of control OCT activity. It should be noted that spf-ash mice have a kinetically normal OCT, but no mutant OCT with a higher K_m value for Orn, as was found in spf

mice [4]. It has been reported that the hyperammonemia and orotic aciduria of a patient with OCT deficiency and kinetically abnormal OCT were ameliorated by a continuous supplement of arginine [42], and that the hyperammonemia and orotic aciduria of spf mice can be normalized by treatment with 5-FMOrn [24] which inactivates OAT and increases Orn concentration beyond the high K_m value of the mutant OCT. The effectiveness of 5-FMOrn on the hyperammonemia of spf-ash mice implies that the OCT reaction is not saturated with Orn in vivo, and strongly suggests that the increase in Orn in vivo following an ammonia load may play a physiological role. Actually, the present study shows a significant reciprocal relationship between ammonia and Orn concentrations under the conditions stated in Fig. 5. From the results presented in this study, we propose that supplying urea cycle intermediate amino acids to patients with partial OCT deficiency is clinically beneficial, and that OAT inhibitors such as 5-FMOrn may be even more effective.

Acknowledgements

We thank Dr. C. Fujiyama for advice on statistical analysis, Mr. M. Gore for editorial assistance, Mr. T. Minato for technical assistance and Ms. M. Tanaka for secretarial assistance. This research was supported by the Kodama Memorial Fund for Medical Research.

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